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# Species-Specific Identification of and Distinction between *Borrelia burgdorferi* Genomic Groups by Using 16S rRNA-Directed Oligonucleotide Probes

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**Examination of a number of previously published aligned *Borrelia* 16S rRNA sequences revealed the presence of regions which could serve as oligonucleotide probe targets for both species-specific identification of *Borrelia burgdorferi* and distinction between genomic groups. Total cellular RNA isolated from *Borrelia* cultures was used in slot blot analysis. Radiolabeled oligonucleotides designed to hybridize to specific 16S rRNA targets were used as probes. These probes allowed for both species-specific identification and genomic group typing of *B. burgdorferi*.**

Lyme disease now represents the most frequently diagnosed human tick-borne disease in Europe and North America (17, 20). *Borrelia burgdorferi*, the causative agent of Lyme disease, is transmitted to a wide range of vertebrates (8) via the bite of infected ticks of the *Ixodes ricinus* complex. In addition to *B. burgdorferi*, the genus *Borrelia* contains several additional species which are tick borne (for a review, see references 4 and 11). Until recently, *Borrelia* species were identified solely upon the geographic region and arthropod host from which they were isolated. More recently, the approach to identification has focused on reactivity with species-specific monoclonal antibodies (5, 7, 27). This approach, however, is limited by the antigenic variation seen among isolates (6, 31, 32) and by in vitro cultivation (25, 29). Antigenic variation is of particular concern with regard to the outer surface proteins, OspA and OspB. Substantial antigenic and size variations of these proteins are well documented (7). The polymerase chain reaction has also been successfully used for the identification of *B. burgdorferi* (13, 15, 17, 22).

Significant genetic divergence among isolates was first suggested by differences in monoclonal antibody reactivity (6), plasmid profiles (26), and the clinical manifestations of Lyme disease in Europe versus North America (28). As a result of these observations, considerable emphasis has been focused on the comparison of European and North American isolates (18, 19, 20, 23). In general, European isolates exhibit greater heterogeneity than North American isolates with respect to several criteria, including monoclonal antibody reactivity (6) and amplification with various polymerase chain reaction primers (24). In a survey of 12 different isolates, including both European and North American representatives, Postic and coworkers identified two genomic groups of *B. burgdorferi* by using DNA-DNA hybridization studies and Southern analysis of rRNA gene restriction patterns (23). These two genomic groups were termed the sensu stricto and 20047 groups. North American isolates reside solely in the sensu stricto group. Most European isolates reside in the 20047 group, but some exceptions have been observed (18, 23, 24). Recently, we investigated the phylogenetic divergence among isolates by using rRNA sequence analysis and rRNA gene restriction profiles (18, 19). The 16S rRNA sequences were determined for several *B. burgdorferi* isolates as well as for *B. hermsii*, *B. coriaceae*, and *B. anserina*. *B. burgdorferi* isolates from Europe, North America, and Russia were included. All isolates from North America (B31, Sh-2-82, and 1352) and isolate 20004 from Europe were found to reside in the sensu stricto group. The remaining European isolates, G2 and G1, were placed in the 20047 group. Recently, we defined a third genomic group (group 3) comprising tick isolates (*I. persulcatus*) from the Soviet Union (19). Alignment of the 16S rRNA sequences revealed the presence of signature nucleotides unique to each genomic group. In addition, signature nucleotides common to all isolates of *B. burgdorferi* (i.e., species specific) were observed.

Using *Borrelia* 16S rRNA sequences, we constructed probes that serve to distinguish *B. burgdorferi* from other *Borrelia* species and to distinguish between the genomic groups of *B. burgdorferi*. Other groups have developed *B. burgdorferi* species-specific probes by using polymerase chain reaction amplification (13, 15, 19, 22). We chose rRNA as the target molecule since it is present in large quantities within a cell, so rRNA targets can be considered to be naturally highly amplified. In addition, rRNA molecules are highly conserved and presumably are subject to a very low mutation frequency. The specificity of the probes was demonstrated through the use of slot blots with total cellular RNA as the target. This approach allows the reliable identification and genomic typing of *B. burgdorferi* from cultures, typically within 36 h.

## MATERIALS AND METHODS

**Bacterial strains and culture conditions.** The isolates and species used in this study are described in Table 1. All *Borrelia* cultures were grown in BSKII medium at 34°C (2, 3) and passaged every 3 to 7 days. Rifampin was added to all *B. burgdorferi* cultures at 50 μg/ml.

**Total cellular RNA isolation, probe design, and 5′ end labeling.** Total cellular RNA was isolated as originally described by Thomas (30), with some modifications (18). Hybridization probes were designed on the basis of a previ-

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TABLE 1. Isolates and species used in this study

| Species | Isolate | Geographic origin | Source | 16S rRNA sequence accession number[a] |
|---|---|---|---|---|
| *B. burgdorferi* | B31 | New York | *I. dammini* | X57404 |
| | Sh-2-82 | New York | *I. dammini* | M60969 |
| | NY13-87 | New York | Human, skin | —[b] |
| | 21721 | Wisconsin | *I. dammini* | — |
| | 21343 | Wisconsin | Mouse | — |
| | 1352 | Texas | *A. americanum* | M64309 |
| | 3028 | Texas | Human, pus | — |
| | CA2-87 | California | *I. pacificus* | — |
| | Veery | Connecticut | Veery bird | — |
| | 20004 | France | *I. ricinus* | M64310 |
| | G2 | Germany | Human, cerebrospinal fluid | M60967 |
| | G1 | Germany | Human, cerebrospinal fluid | M64311 |
| | R-IP21 | Russia (Leningrad) | *I. persulcatus* | — |
| | R-IP3 | Russia (Leningrad) | *I. persulcatus* | — |
| | R-IP90 | Russia (Khabarovsk) | *I. persulcatus* | — |
| | J1 | Japan | *I. persulcatus* | — |
| *B. coriaceae* | Co53 | ? | *O. coriaceus* | M60970 |
| *B. hermsii* | HS1 | California | *O. hermsii* | M60968 |
| | MAN | California | Human, blood | — |
| | YOR | California | Human, blood | — |
| *B. anserina* | BA2 | ? (United States) | ? | M64312 |
| *B. turicatae* | — | ? (United States) | ? | — |
| *E. coli* | DH5 | — | — | — |

[a] GenBank/EMBL databases.
[b] —, only partial sequences have been determined.

ously presented alignment of *Borrelia* 16S rRNA sequences (18). Synthesis, labeling, and purification of oligonucleotides were as previously described (18).

**Slot blot hybridization analysis.** Slot blot hybridization analysis was performed by use of the Mini-fold II system (Schleicher & Schuell) with precut BA85 nitrocellulose membranes. Membranes were pretreated by being soaked in warm distilled $H_2O$ and then in 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate [pH 7.0]). Total cellular RNA was serially diluted over a range of 1 μg/μl to 1 ng/μl, and amounts ranging from 1 μg to 1 ng were combined with 400 μl of a solution of 6.15 M formaldehyde–10× SSC and incubated at 65°C for 15 min. Prior to application of the samples to the membranes, each well was washed with 400 μl of 10× SSC. After sample loading, each well was washed with 400 μl of 10× SSC and allowed to remain under vacuum for 5 min. Membranes were baked for 1 to 2 h under vacuum at 80°C.

Dried membranes were hydrated in 2× SSC and prehybridized in hybridization buffer (10% dextran sulfate [molecular biology grade; molecular weight, 500,000; Sigma Chemical Co.], 0.2% polyvinylpyrrolidone [molecular weight, 360,000; Sigma], 0.2% bovine serum albumin, 0.2% Ficoll [type 400; Sigma], 0.05 M Tris-HCl [pH 7.5], 1.0 M NaCl, 0.1% $NaPP_i$, denatured herring sperm DNA [100 to 150 μg/ml; Promega Biotec]) in glass bottles at 35°C in an Autoblot hybridization oven (Bellco Glass) for 0.5 to 2 h. After prehybridization, the buffer was removed and replaced with 10 ml of fresh buffer containing 100 ng of the labeled probe. Hybridization was performed at 35°C for 4 to 16 h. Following hybridization, the buffer was removed and the membranes were washed in the bottles with 50 ml of 6× SSC–0.1% sodium dodecyl sulfate (SDS) twice for 10 min each time at room temperature and once at 35°C with 50 ml of 1× SSC–0.1% SDS for 30 min. Membranes which were screened with the genomic group-specific probes, G2-84 and G3-84, were washed one additional time with 0.5× SSC–0.1% SDS for 1 h at 37°C. Membranes were blotted dry, wrapped in cellophane, and exposed with intensifying screens at −70°C for 0.5 to 6 h. When genomic group-specific probes G2-84 and G3-84 were used to screen the isolates, detection of a signal in the 1-ng RNA-containing lanes required exposure of the film for ca. 15 h.

## RESULTS AND DISCUSSION

In this study, we sought to develop probes directed against rRNA targets and capable of distinguishing *B. burgdorferi* from other *Borrelia* species as well as of differentiating between the previously established genomic groups of *B. burgdorferi* (18, 19, 23). Species-specific identification has been achieved primarily through reactivity with monoclonal antibodies (5). Distinction between *B. burgdorferi* genomic groups has been achieved by Southern analysis, probing for rRNA gene patterns (18, 23), DNA-DNA hybridization studies (23), or sequencing of rRNA and phylogenetic analysis (18, 19). While these approaches have proven valuable in assessing phylogenetic relationships, they are somewhat laborious and time-consuming. Oligonucleotide probes directed against rRNA targets have been used by several groups for the detection, species identification, and typing of isolates of several different organisms, including *Mycoplasma* (12), *Treponema* (14), *Campylobacter* (10), *Haemophilus* (21), and *Clostridium* (33) spp.

A

| | | |
|---|---|---|
| *B. burgdorferi* | 631 | |
| 1) *sensu stricto* | CUAUGUUGGA AACUAUAUGU CU | 3' |
| 2) *group 20047* | ..... .....a. .. | |
| 3) *group 3* | ..... ..... .. | |
| *B. hermsii* | a....c.... ....gc...a .. | |
| *B. anserina* | .....c.a.. ....gc...a .. | |
| *B. coriaceae* | .....c.... ....gc...a .. | |
| Probe 631 B | GATACAACCT TTGATATACA GA | 5' |
| Probe 631 H | GATACGACCT TTGACGTACT GA | 5' |

B

| | | |
|---|---|---|
| *B. burgdorferi* | 181 | |
| 1) *sensu stricto* | AAGGUCAGUU AAUUUGUUA | 3' |
| 2) *group 20047* | ..a....a.. ..... | |
| 3) *group 3* | .....a.. ..... | |
| Probe G1-181 | TTCCAGTCAA TTAAACAAT | 5' |

C

| | | |
|---|---|---|
| *B. burgdorferi* | 84 | |
| 1) *sensu stricto* | AAUACAUUCA GUGGC | 3' |
| 2) *group 20047* | .....cu. ..... | |
| 3) *group 3* | .....u. ..... | |
| Probe G2-84 | TTATGTAGAT CACCG | 5' |
| Probe G3-84 | TTATGTAAAT CACCG | 5' |

FIG. 1. 16S rRNA probe target sites. 16S rRNA *Borrelia* sequences corresponding to the oligonucleotide probe target sites are aligned and compared with the oligonucleotide probe sequences. For *B. burgdorferi*, the consensus sequence for each genomic group is given (18, 19). Isolates belonging to each of the groups are described in the text. The sequences for isolates belonging to the sensu stricto group are given in each panel. Positions which differ in other isolates or species are indicated by lowercase lettering. Positions of homology are indicated by dots. Probe sequences are listed in the opposite orientation, i.e., 3′ to 5′, to demonstrate their complementarity to the rRNA. (A) Base-pair alignment of the 631B probe (*B. burgdorferi* species specific) and the 631H probe. (B) Base-pair alignment of the G1-181 genomic group-specific probe. (C) Base-pair alignment of probes G2-84 and G3-84.

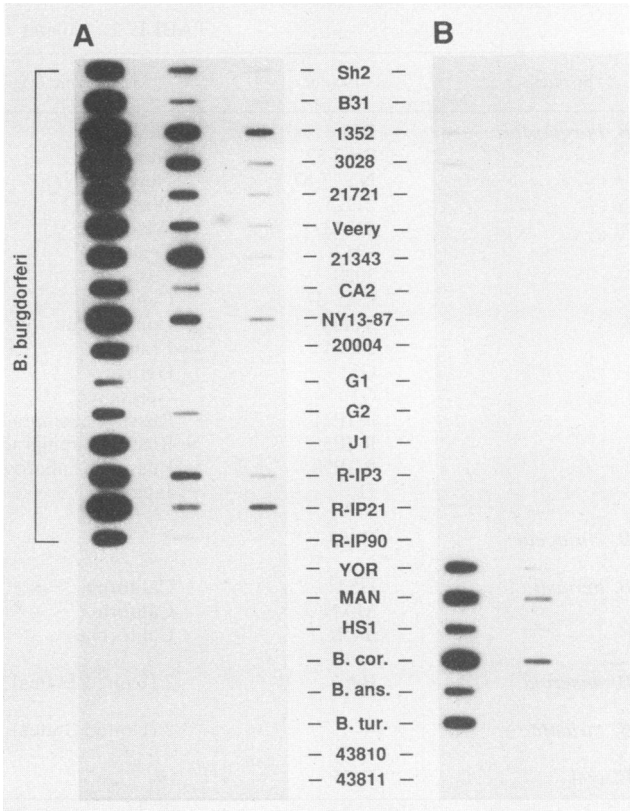


FIG. 2. Slot blot analysis with the *B. burgdorferi* species-specific 631B probe. Slot blots of total cellular RNA were prepared and hybridized as described in Materials and Methods. (A) Blots were probed with the *B. burgdorferi* 631B probe. (B) The blot from panel A was stripped of the probe by being boiled for 1 min in DEPC-$H_2O$, slowly cooled to room temperature, and reprobed with probe 631H. YOR, MAN, and HS1 are *B. hermsii* isolates. B. cor., *B. coriaceae*; B. ans., *B. anserina*; B. tur., *B. turicatae*. 43810 and 43811 are ATCC *Spirochaeta* isolates.

The rRNA regions chosen for hybridization sites and the probes used are indicated in Fig. 1. It had been shown previously that selective hybridization of a probe to a given target can be achieved when potential competing target sequences differ by as little as one base (9). Species-specific detection of *B. burgdorferi* was accomplished by an oligonucleotide probe directed against the rRNA region spanning bases 631 to 652 (*Escherichia coli* nomenclature). On the basis of earlier sequencing results, this target site contains four positions which are diagnostic for *B. burgdorferi* (Fig. 1) and which distinguish it from other *Borrelia* species (18). We tested a total of 16 different isolates of *B. burgdorferi*. The isolates chosen included representatives from widely distant geographic regions and both human and tick sources. Furthermore, the isolates chosen included representatives of the three genomic groups (18, 19). All 16 isolates of *B. burgdorferi* hybridized with the labeled 631B probe (Fig. 2A). The reduced signal observed for isolates G1 and G2 can be attributed to the presence of a single base mismatch within the rRNA target sequence (18). Other minor differences in signal intensity may be due to any of several factors, including slight differences in the actual total amount of RNA loaded, differences in the degree of denaturation of the templates, and differences in the amount of degradation of the RNA target. All of the non-*B. burgdorferi* isolates were negative for hybridization. These included *B. coriaceae*, *B. anserina*, *B. hermsii* (three different isolates), *B. turicatae*, *E. coli* (data not shown), and *Spirochaeta* isolates not identified to the species level (ATCC 43810 and ATCC 43811, from *Peromyscus leucopus* and *Blarina brevicauda*, respectively) (1). A review of published 16S rRNA sequences did not reveal any significantly homologous sequences in other eubacteria, plastids, archae, or eukaryotes.

A second probe (631H), also directed to the region spanning bases 631 to 652 but designed to hybridize with the other *Borrelia* species (i.e., *B. anserina*, *B. hermsii*, and *B. coriaceae*), was synthesized and used in a slot blot analysis as a control to demonstrate that the above-mentioned negative hybridization results were not due to degradation of the templates. The 631H target site sequence is highly conserved among *Borrelia* species carried by soft-bodied ticks. This probe yielded positive hybridization results with all *Borrelia* species other than *B. burgdorferi* (Fig. 2B). The non-*Borrelia* species tested were also negative. A minor amount of signal (approximately 100-fold lower than that of the other *Borrelia* species) could be detected with some *B. burgdorferi* isolates. This signal most likely reflects incomplete stripping of the 631B probe. Nonetheless, the relative differences in the signals allow easy distinction between positive and negative results. Since both *B. anserina* and *B. coriaceae* are noninfectious for humans, the 631H probe may provide a means of identifying relapsing fever spirochetes in cultures for human patients. In contrast to *B. burgdorferi*, *B. hermsii* can be readily isolated from the blood of infected individuals.

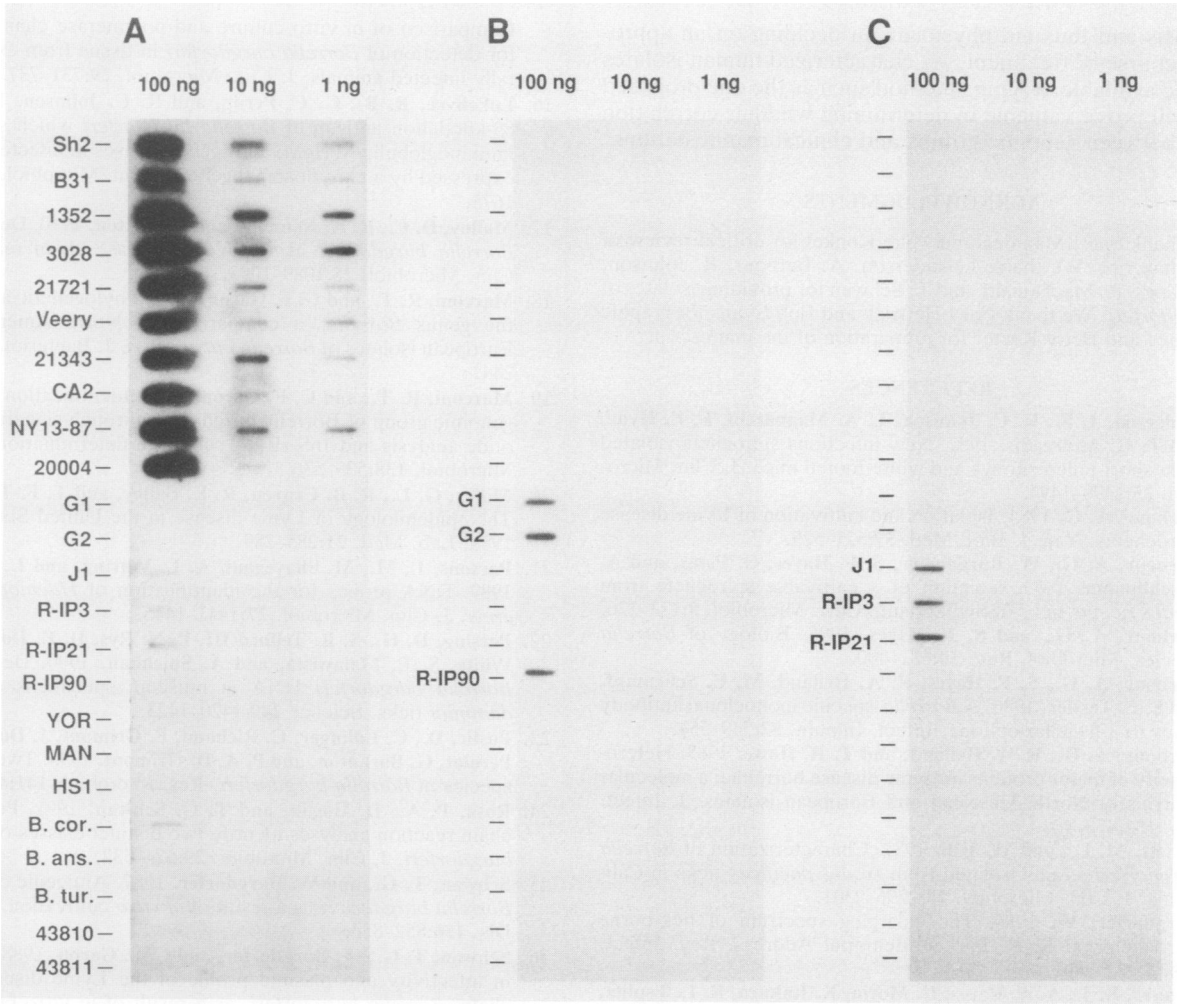


FIG. 3. Slot blot analysis with genomic group-specific probes. Slot blots were prepared and hybridized as described in Materials and Methods. Blots were probed with probe G1-181, G2-84, or G3-84 (A, B, and C, respectively). Total cellular RNA (100, 10, and 1 ng, from left to right) from each isolate was used as the hybridization target. Isolates were as described in the legend to Fig. 2.

There are currently three recognized genomic groups of *B. burgdorferi*, termed sensu stricto, 20047, and 3 (18, 19, 23). Three probes were designed to allow distinction between these groups (Fig. 1). Probe G1-181 complements the rRNA sequence spanning residues 181 to 199 of members of the sensu stricto genomic group. Probes G2-84 and G3-84 both complement the 16S rRNA region spanning bases 84 to 98 of genomic groups 20047 and 3, respectively. In a slot blot hybridization analysis, isolates Sh-2-82, B31, 3028, 1352, 21721, 21343, CA2, NY13-87, 20004, and Veery were hybridization positive with the G1-181 probe, suggesting their inclusion in the sensu stricto genomic group (Fig. 3). With the exception of isolate 20004, which was previously assigned to the sensu stricto genomic group, all were of North American origin. By using 16S rRNA signature nucleotide analysis, we confirmed the assignment of these isolates to the sensu stricto genomic group (data not shown). Isolates R-IP3, R-IP21, R-IP90, G1, and G2, all of non-North American origin, were negative for hybridization with the G1-181 probe, demonstrating their exclusion from the sensu stricto genomic group.

Probe G3-84, which was designed to identify members of group 3, hybridized with isolates J1, R-IP3, and R-IP21. Isolates R-IP3 and R-IP21 were the first isolates of group 3 to be identified (19). These isolates were found to be members of a third genomic group on the basis of 16S rRNA sequence analysis. The inclusion of isolate J1 in genomic group 3 was confirmed by determination of the signature nucleotide composition with primers previously designed for this purpose (19). Isolate J1 thus represents the third isolate to be placed in this group. When probe G2-84, which was designed to hybridize with isolates of group 20047, was used to screen the isolates, G1, G2, and R-IP90 were found to be positive. Isolates G1 and G2 were previously identified as members of this group (18). Isolate R-IP90, a tick isolate from the Soviet Union, was hybridization positive, demonstrating its inclusion in this group.

Although it is now clear that *B. burgdorferi* isolates can be typed into distinct genomic groups, the significance of these groups with respect to the clinical presentations of Lyme borreliosis patients remains unclear. A correlation between genomic group and the resulting disease process remains elusive, primarily because of the current unavailability of characterized human isolates for which well-documented case histories are available. Should such a correlation exist, knowledge of a genomic type may allow for a more precise

diagnosis and thus aid physicians in deciding on an appropriate course of treatment. As characterized human isolates become available, a typing method such as the one proposed here will prove valuable in determining whether correlation exists between genomic groups and clinical manifestations.

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